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14. ABSTRACT
Using a novel mouse model to introduce oncogenes to murine somatic mammary epithelial cells, I have found that acute expression of the ErbB2 oncogene can induce a robust DNA damage response in premalignant lesions, similar to what has been observed in clinical breast samples. This response results in p53 stabilization, apoptosis, and senescence and requires intact ATM function. Furthermore, during sporadic ErbB2 tumor evolution, p53 stabilization and apoptosis are lost, indicating these are critical tumor suppressive mechanisms in curtailing ErbB2 tumorigenesis. However, senescence remains in ErbB2 tumors, suggesting that it may not be a critical barrier to tumorigenesis.

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Introduction

Double strand breaks (DSBs) in chromosomal DNA are recognized by the MRN complex, which recruits the serine/threonine kinase ataxia-telangiectasia mutated (ATM). Upon autophosphorylation and activation, ATM phosphorylates the histone variant H2AX as well as additional factors (e.g. MDC1, 53BP1, and BRCA1) that are also recruited to DSBs [1, 2]. These adaptor proteins serve to reinforce ATM signaling in a positive feedback loop that culminates in the phosphorylation and activation of transducer kinases, Chk1 and Chk2, which, in addition to ATM, will phosphorylate, stabilize, and activate effector proteins, the most critical one being p53 [3]. If DNA DSBs cannot be repaired in a timely fashion, p53 will halt cell proliferation by inducing either apoptosis or senescence [4].

DNA replicative stress imposed by oncogene-induced aberrant cell proliferation induces DSBs, and components of this DNA damage response (DDR) signaling cascade have been found in the early, hyperplastic lesions in several tissues, including the breast [5, 6]. However, apoptosis and senescence are rarely found in tumors, and this has been suggested to be due to the disruption of DDR signaling caused by mutations in genes encoding p53 and other mediators of a DDR [7, 8]. Indeed, *p53, ATM, Chk2, BRCA1, 53BP1, and MDC1* are mutated or inactivated in many human malignancies including breast cancer [6, 9-15]. *ATM* nullizygosity also predisposes mice to thymic lymphomas [16-20]. *ATM* inactivation accelerates c-Myc-induced carcinogenesis and reduces c-Myc-induced apoptosis in both Eμ-*c-Myc* B-cell lymphoma and keratin 5-*c-Myc* squamous cell carcinoma transgenic mouse models [21, 22], and *ATM* heterozygosity promotes mammary tumorigenesis induced by DMBA or *p53* heterozygosity [23, 24]. In cultured cells and xenograft models, ATM and subsequent DDR activation is required for senescence induced by Ras and several other mitogenic factors [25-27]. Therefore, a DDR and subsequent apoptosis and senescence likely represent a critical barrier to carcinogenesis [7, 28].

However, the DDR oncogenic barrier may not operate in all tissues. For example, *ATM* was found to be dispensable for p53-dependent apoptosis in a mouse model of choroid plexus tumorigenesis [29], and for oncogene-induced DDR induction, senescence, and p53-dependent tumor suppression in *K-Ras*-driven lung carcinoma and chemically-induced fibrosarcoma mouse models [30]. In addition, ATM was not necessary for p53-dependent inhibition of tumorigenesis in a mouse model of chemically-induced skin carcinoma [31].

Aberrant activation of growth factor signaling occurs very frequently in breast cancer. For example, *ErbB2 (HER2, Neu)*, encoding a member of the epidermal growth factor receptor family of receptor tyrosine kinases, is amplified in 20-30% of human breast cancers [32, 33]. The genes encoding ErbB1, ErbB3, and insulin-like growth factor receptors are also sometimes overexpressed or activated in breast cancers [34-36]. Aberrant activation of growth factor signaling leads to rapid cell proliferation in cultured cells, yet these genes alone are insufficient to induce breast cancer in animal models. An ATM-p53 DDR signaling cascade may be acting to prevent these oncogenes from inducing mammary tumors, but this hypothesis has not yet been tested in vivo.

In our proposed work, we sought to gain insights in the roles of DDR signaling in breast carcinogenesis by using both established and novel mouse models of breast cancer. Our preliminary efforts revealed that that retrovirus-mediated expression of the gene encoding polyoma middle T antigen (*PyMT*) in somatic mammary epithelial cells induces preneoplastic lesions with evidence of DDR signaling, p53 stabilization, and apoptosis. Such effects could not be observed when using several, established transgenic mouse models of mammary cancer. Additionally, somatic *ErbB2* activation induces these responses as well as senescence. However, in the tumors that form as a result of somatic ErbB2 activation, p53 stabilization and apoptosis were absent, indicating that DDR signaling becomes compromised. Collectively, these findings led to my hypothesis that the **DDR represents a critical barrier to ErbB2-mediated breast tumorigenesis *in vivo* and must be inactivated for cancer to progress.**

Body

Training

Undertaking my graduate training at the Lester and Sue Smith Breast Center at Baylor College of Medicine (BCM) has been an invaluable learning experience. Under the direction of my mentor, Yi Li, Ph.D., I have had myriad opportunities to design and conduct cutting-edge research in the field of molecular breast oncology using the latest advances in mouse genetics and molecular biology. A key aspect of training the BCM Breast Center remains many opportunities to present one's work to peers and renowned faculty. At various lab meetings, seminars, symposia, and retreats I have presented my findings and gained valuable feedback, which are instrumental in advancing my project and my training as a scientist. In addition, I have had numerous opportunities to give and attend journal clubs where trainees and faculty discuss high profile, recently published papers in the breast cancer field. Finally, I have attended numerous seminars by faculty from all over the world who have come to BCM to present their most recent findings.

Research Progress

Of the three tasks or specific aims outlined in my initial proposal, the majority of the progress has been made on Tasks 1 and 2. These findings have recently been published [37] and will be briefly summarized below. For detailed aspects of this research, please reference this manuscript (attached as Appendix A).

Task 1: Determine the mechanism for DDR inactivation in ErbB2-induced mammary tumors.

My lab has previously reported the use of a retroviral delivery system to introduce various oncogenes, including *ErbB2*, into the in vivo somatic mammary epithelium of mice engineered to express the glycoprotein tva [38]. I have recently published an overview of this mouse model [39], which is attached (Appendix B). In the case of ErbB2-induced tumors, I found preliminary evidence that p53 stabilization and apoptosis were absent, suggesting the tumor suppressive DNA damage response may have been inactivated during sporadic ErbB2 tumorigenesis. Since these initial findings, I have confirmed that p53 stabilization and apoptosis are indeed lost in advanced mammary tumors arising as a result of acute ErbB2 activation. However, qRT-PCR analysis demonstrated that *p53* mRNA was present in these ErbB2 tumors and at higher levels than in irradiated splenic controls. Upon further investigation, we found that γ-irradiation of ErbB2 tumors results in the upregulation of p53 target genes *p21* and *Mdm2*, as assessed by qRT-PCR, indicating that the *p53* genetic locus can regulated appropriately. Furthermore, activated p53 (as assessed by Western blotting with phospho-serine 15-p53 antibodies) was observed after ErbB2 tumor irradiation. These findings collectively indicate that the p53 genetic locus in ErbB2 tumors is intact and can generate functional protein product upon exposure to ionizing radiation. Thus, p53 is likely misregulated at the post-translational, and not at the genetic, level during ErbB2 tumorigenesis, which allows for the loss of apoptosis and subsequent malignant progression. As mentioned previously, these results have been recently published [37] and are attached as Appendix A.

Task 2: Determine whether the loss of a functional DDR results in increased tumor formation.

In order to determine the importance of DDR function in curtailing somatic ErbB2 tumorigenesis, I employed an in vivo genetic ablation strategy. I mated ATM heterozygous mice [17] to MMTV-TVA mice [38]. The resulting offspring were then interbred to generate ATM-null, MMTV-TVA mice that would be susceptible to infection by retroviruses carrying *ErbB2*. My initial experimental design was to induce ErbB2 activation in $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mice and monitor for tumor formation. However, $Atm^{-/-}$ mice have high incidence thymic lymphomas and do not survive long enough to form mammary tumors [16-20]. In light of this, I examined the premalignant lesions that arise all three genotypes of mice as a result of acute ErbB2 activation. I found that ATM loss results in the ablation of the DNA damage response that is typically

mounted in response to ErbB2 activation and ensuing replication stress. Furthermore, there was a reduction in p53 stabilization, apoptosis, and senescence upon ATM loss. These findings collectively indicated that ATM, and consequently DNA damage signaling, represents a critical brake on ErbB2-driven tumorigenesis. As mentioned above, these results were recently published [37] and are attached as Appendix A.

To directly test whether ATM loss will result in increased ErbB2 tumorigenesis, alternative strategies are required. The ideal experimental plan would be to ablate ATM function specifically in the mammary epithelium using a mammary-specific hit-and-run Cre recombinase on a loxP-flanked ATM allele. However, no such alleles or tissue-specific ATM knockout mice have been generated to our knowledge, requiring our lab to make them.

Task 3: Determine whether specific mammary cell subtypes are better able at circumventing the DDR in mammary carcinogenesis.

No progress has yet been made on this aim.

Key Research Accomplishments:

- Confirmed that DDR signaling induced by acute ErbB2 activation in somatic mammary epithelial cells activates tumor suppressive programs, including p53 stabilization, apoptosis, and senescence.
- Demonstrated that these tumor suppressive mechanisms are dependent on ATM function, using in vivo mouse genetics.
- Demonstrated that ErbB2-induced mammary tumorigenesis displays loss of p53 stabilization and apoptosis, indicating that these are critical components in ErbB2 tumor suppression.

Reportable Outcomes:

- Invited to present results at BCM Molecular & Cellular Biology Annual Symposium, Houston, TX.
- Presented poster at 24th Annual MD/PhD Student Conference, Keystone, CO
- Invited to present results at BCM Breast Center Annual Retreat, Houston, TX.
- Awarded AACR Scholar-in-Training award to present results at AACR Frontiers in Basic Cancer Research, Boston, MA.
- Awarded AACR Scholar-in-Training award to present results at AACR Advances in Breast Cancer Research conference, San Diego, CA.
- Published paper on the use of RCAS-TVA technology as a method to introduce oncogenes into murine somatic mammary epithelial cells in vivo (Reddy, J.P. and Y. Li, *The RCAS-TVA System for Introduction of Oncogenes into Selected Somatic Mammary Epithelial Cells in Vivo.* J Mammary Gland Biol Neoplasia, 2009).
- Published results (Reddy, J.P., et al., *Defining the ATM-mediated barrier to tumorigenesis in somatic mammary cells following ErbB2 activation.* Proc Natl Acad Sci U S A).

Conclusion

Upon completion of the first year of this pre-doctoral fellowship, I have made significant progress towards my project goals of better delineating the role of the DNA damage response in breast cancer initiation and progression. I have determined that acute oncogene expression in murine somatic mammary epithelial can engender a robust DDR that has been previously observed in human breast clinical samples but not in established mouse models of mammary cancer. Furthermore, I have determined that the well-established human breast cancer oncogene, ErbB2, induces a DDR during cancer initiation, which is critical for anti-tumor barriers to be erected in response to ErbB2 activation and must be inactivated for advanced ErbB2 tumors to form. Hopefully, these insights will extend our understanding into the etiology of ErbB2 breast tumors and lead to the development of novel therapeutic strategies designed to

bolster DDR signaling which may be of potential benefit in breast cancer prevention and treatment.

References:

1. Kastan, M.B., *DNA damage responses: mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture.* Mol Cancer Res, 2008. **6**(4): p. 517-24.
2. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.
3. Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer.* Nature, 2004. **432**(7015): p. 316-23.
4. d'Adda di Fagagna, F., *Living on a break: cellular senescence as a DNA-damage response.* Nat Rev Cancer, 2008. **8**(7): p. 512-22.
5. Gorgoulis, V.G., et al., *Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions.* Nature, 2005. **434**(7035): p. 907-13.
6. Bartkova, J., et al., *DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis.* Nature, 2005. **434**(7035): p. 864-70.
7. Halazonetis, T.D., V.G. Gorgoulis, and J. Bartek, *An oncogene-induced DNA damage model for cancer development.* Science, 2008. **319**(5868): p. 1352-5.
8. Evan, G.I. and F. d'Adda di Fagagna, *Cellular senescence: hot or what?* Curr Opin Genet Dev, 2009.
9. Bartkova, J., et al., *DNA damage response mediators MDC1 and 53BP1: constitutive activation and aberrant loss in breast and lung cancer, but not in testicular germ cell tumours.* Oncogene, 2007. **26**(53): p. 7414-22.
10. Prokopcova, J., et al., *The role of ATM in breast cancer development.* Breast Cancer Res Treat, 2007. **104**(2): p. 121-8.
11. Pylkas, K., et al., *Evaluation of the role of Finnish ataxia-telangiectasia mutations in hereditary predisposition to breast cancer.* Carcinogenesis, 2007. **28**(5): p. 1040-5.
12. Renwick, A., et al., *ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles.* Nat Genet, 2006. **38**(8): p. 873-5.
13. Rio, P.G., et al., *Loss of heterozygosity of BRCA1, BRCA2 and ATM genes in sporadic invasive ductal breast carcinoma.* Int J Oncol, 1998. **13**(4): p. 849-53.
14. Tommiska, J., et al., *The DNA damage signalling kinase ATM is aberrantly reduced or lost in BRCA1/BRCA2-deficient and ER/PR/ERBB2-triple-negative breast cancer.* Oncogene, 2008. **27**(17): p. 2501-6.
15. Zhang, Y., Y. Xiong, and W.G. Yarbrough, *ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways.* Cell, 1998. **92**(6): p. 725-34.
16. Barlow, C., et al., *Atm-deficient mice: a paradigm of ataxia telangiectasia.* Cell, 1996. **86**(1): p. 159-71.
17. Borghesani, P.R., et al., *Abnormal development of Purkinje cells and lymphocytes in Atm mutant mice.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3336-41.
18. Elson, A., et al., *Pleiotropic defects in ataxia-telangiectasia protein-deficient mice.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 13084-9.
19. Herzog, K.H., et al., *Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system.* Science, 1998. **280**(5366): p. 1089-91.
20. Xu, Y., et al., *Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma.* Genes Dev, 1996. **10**(19): p. 2411-22.
21. Reimann, M., et al., *The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo.* Blood, 2007. **110**(8): p. 2996-3004.

22. Pusapati, R.V., et al., *ATM promotes apoptosis and suppresses tumorigenesis in response to Myc.* Proc Natl Acad Sci U S A, 2006. **103**(5): p. 1446-51.
23. Lu, S., et al., *Atm-haploinsufficiency enhances susceptibility to carcinogen-induced mammary tumors.* Carcinogenesis, 2006. **27**(4): p. 848-55.
24. Umesako, S., et al., *Atm heterozygous deficiency enhances development of mammary carcinomas in p53 heterozygous knockout mice.* Breast Cancer Res, 2005. **7**(1): p. R164-70.
25. Bartkova, J., et al., *Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints.* Nature, 2006. **444**(7119): p. 633-7.
26. Di Micco, R., et al., *Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication.* Nature, 2006. **444**(7119): p. 638-42.
27. Mallette, F.A., M.F. Gaumont-Leclerc, and G. Ferbeyre, *The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence.* Genes Dev, 2007. **21**(1): p. 43-8.
28. Venkitaraman, A.R., *Medicine: aborting the birth of cancer.* Nature, 2005. **434**(7035): p. 829-30.
29. Liao, M.J., et al., *Atm is dispensable for p53 apoptosis and tumor suppression triggered by cell cycle dysfunction.* Mol Cell Biol, 1999. **19**(4): p. 3095-102.
30. Efeyan, A., et al., *Limited role of murine ATM in oncogene-induced senescence and p53-dependent tumor suppression.* PLoS One, 2009. **4**(5): p. e5475.
31. Bailey, S.L., et al., *Tumor suppression by p53 in the absence of Atm.* Mol Cancer Res, 2008. **6**(7): p. 1185-92.
32. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-82.
33. Slamon, D.J., et al., *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer.* Science, 1989. **244**(4905): p. 707-12.
34. Chan, S.K., M.E. Hill, and W.J. Gullick, *The role of the epidermal growth factor receptor in breast cancer.* J Mammary Gland Biol Neoplasia, 2006. **11**(1): p. 3-11.
35. Hamburger, A.W., *The role of ErbB3 and its binding partners in breast cancer progression and resistance to hormone and tyrosine kinase directed therapies.* J Mammary Gland Biol Neoplasia, 2008. **13**(2): p. 225-33.
36. Jin, Q. and F.J. Esteva, *Cross-talk between the ErbB/HER family and the type I insulin-like growth factor receptor signaling pathway in breast cancer.* J Mammary Gland Biol Neoplasia, 2008. **13**(4): p. 485-98.
37. Reddy, J.P., et al., *Defining the ATM-mediated barrier to tumorigenesis in somatic mammary cells following ErbB2 activation.* Proc Natl Acad Sci U S A.
38. Du, Z., et al., *Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17396-401.
39. Reddy, J.P. and Y. Li, *The RCAS-TVA System for Introduction of Oncogenes into Selected Somatic Mammary Epithelial Cells in Vivo.* J Mammary Gland Biol Neoplasia, 2009.

Defining the ATM-mediated barrier to tumorigenesis in somatic mammary cells following ErbB2 activation

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p53, apoptosis, and senescence are frequently activated in preneoplastic lesions and are barriers to progression to malignancy. These barriers have been suggested to result from an ATM-mediated DNA damage response (DDR), which may follow oncogene-induced hyperproliferation and ensuing DNA replication stress. To elucidate the currently untested role of DDR in breast cancer initiation, we examined the effect of oncogene expression in several murine models of breast cancer. We did not observe a detectable DDR in early hyperplastic lesions arising in transgenic mice expressing several different oncogenes. However, DDR signaling was strongly induced in preneoplastic lesions arising from individual mammary cells transduced in vivo by retroviruses expressing either *PyMT* or *ErbB2*. Thus, activation of an oncogene after normal tissue development causes a DDR. Furthermore, in this somatic ErbB2 tumor model, ATM, and thus DDR, is required for p53 stabilization, apoptosis, and senescence. In palpable tumors in this model, p53 stabilization and apoptosis are lost, but unexpectedly senescence remains in many tumor cells. Thus, this murine model fully recapitulates early DDR signaling; the eventual suppression of its endpoints in tumorigenesis provides compelling evidence that ErbB2-induced aberrant mammary cell proliferation leads to an ATM-mediated DDR that activates apoptosis and senescence, and at least the former must be overcome to progress to malignancy. This in vivo study also uncovers an unexpected effect of ErbB2 activation previously known for its prosurvival roles, and suggests that protection of the ATM-mediated DDR-p53 signaling pathway may be important in breast cancer prevention.

oncogene | DNA damage response | p53 | apoptosis | senescence

Apoptosis and senescence are frequently found in precancerous lesions but are rarely detected in cancerous tissues (1). These cellular responses in mutated, precancerous cells have been suggested to be oncogene-activated barriers to tumorigenesis that prevent progression to malignancy. Accordingly, these barriers must be inactivated before cancer can arise (2). The p53 tumor suppressor, which is frequently associated with apoptosis and senescence, is a key player in halting progression to cancer and often inactivated in tumors (3). Activation of a few oncogenes, such as *c-Myc* and *Ras*, is known to cause apoptosis and/or senescence through the activation of ARF, leading to p53 accumulation (3). However, more recently it has been suggested that a DNA damage response (DDR) pathway follows oncogene-induced aberrant cell proliferation and is responsible for p53 stabilization, apoptosis, and senescence (4). This response is caused by DNA replication stress, replication fork collapse, and double strand breaks (DSBs) that may follow hyperproliferation, leading to the recruitment of the serine-threonine kinase ataxia-telangiectasia mutated (ATM) to the damaged chromosomal sites (4). ATM phosphorylates the histone variant H2AX (hereafter termed γH2AX) and p53 binding protein 1 (53BP1), which are also recruited to DSBs (5, 6). ATM also directly phosphorylates p53 and indirectly regulates p53 phosphorylation by activating Chk2 and Chk1 (7). Components of this DDR signaling pathway are activated in preneoplastic lesions but are mutated or inactivated in several cancers, including breast cancer, suggesting that the DDR must be overcome during the process of tumorigenesis (8, 9). In support of this, activation of c-Myc has been reported to induce DDR signaling and ATM-dependent apoptosis in skin and hematopoietic cells (10, 11). Activation of *Ras* and several other oncogenes have been found to induce DDR and ATM-dependent senescence in cultured cells (12–14). However, it has not been tested whether an oncogene-induced DDR is required for senescence observed in preneoplastic lesions in vivo. It is also not known whether the DDR plays a critical role in inducing apoptosis in the initiation of epithelial cancers in tissues besides the skin. Furthermore, in several tissues, oncogene activation either fails to induce a DDR or the resulting DDR fails to induce oncogenesis barriers. For example, *ATM* was reported to be dispensable for p53-dependent apoptosis in a murine model of choroid plexus tumorigenesis (15), and for oncogene-induced DDR induction, senescence, and *p53*-dependent tumor suppression in both *K-Ras*-driven lung carcinoma and chemically induced fibrosarcoma murine models (16).

In breast carcinogenesis, apoptosis and senescence were detected in preneoplastic breast lesions in rodent models of breast tumor formation induced by *Ras* or *ErbB2* (17, 18). However, it is not known whether DDR signaling is activated in the mammary gland at any point after oncogene activation and is responsible for the induction of apoptosis or senescence in premalignant lesions. The only potential supporting evidence thus far has been the increased levels of γH2AX and phospho-Chk2 following ectopic expression of *Wnt-1* in cultured mammary epithelial cells (19) and promotion of *p53* heterozygous or DMBA-initiated mammary tumorigenesis by *ATM* heterozygosity (20, 21).

In this study, we surveyed various murine models of breast cancer to gain insight into the possible role of DDR signaling in breast carcinogenesis. We found that the retrovirus RCAS-mediated expression of oncogenes in somatic mammary epithelial cells (22), which more closely mimics human cancer initiation than conventional models, induces a potent DDR that is required for p53 stabilization, apoptosis, and senescence. Furthermore, we found persistent senescence in fully developed tumors, directly challenging the long-standing assumption that both senescence and apoptosis must be inactivated before tumor formation.

Author contributions: J.P.R., J.M.R., L.A.D., and Y.L. designed research; J.P.R., S.P., W.B., and J.Z. performed research; S.H., Y.-C.N.D., K.P., and L.A.D. contributed new reagents/analytic tools; J.P.R., S.P., and Y.L. analyzed data; and J.P.R. and Y.L. wrote the paper.

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Results

Poor DDR Induction in Transgenic Mouse Models of Mammary Cancer. We surveyed several murine models of mammary cancer for evidence of DDR induction. Mammary glands from female mice transgenic for *ErbB2* ($n = 4$), *c-Myc* ($n = 4$), *H-RasG12D* ($n = 3$), or *PyMT* (encoding the polyoma middle T antigen) ($n = 4$) developed early, precancerous lesions by the age of 7–9 weeks, which were harvested and stained for several DDR markers, γH2AX, 53BP1, and p-S1981-ATM. We found no γH2AX focal staining in MMTV-*PyMT* early lesions and only modest induction of γH2AX foci in the lesions from MMTV-*ErbB2*, *-Myc*, and *-H-RasG12D* mice (Fig. 1). Furthermore, there was little 53BP1 and phospho-ATM focal staining in any of the transgenic lines examined (Fig. 1). In addition, we observed no p53 staining in the hyperplastic lesions of any of the transgenic mice examined (Fig. 1). These observations indicate that the DDR is poorly activated in the early lesions of germline transgenic mouse models of mammary cancer.

To test whether mammary cells in a transgenic mouse are still capable of mounting a DDR in response to classical stimuli, we irradiated three 8-week-old MMTV-*PyMT* mice with 6 Gy of ionizing radiation and assayed the mammary glands for DDR markers 30 min later. Although γH2AX foci were detected, the intensity was much less as compared to irradiated, nontransgenic control mammary glands (Fig. S1). This reduction was observed both in relatively benign ducts and in the precancerous early lesions in the transgenic glands, indicating that the mammary epithelium in MMTV-*PyMT* mice possesses a weaker DDR as compared with wild-type mice. A similar pattern was observed when 53BP1 and p-S1981-ATM were examined by immunofluorescence (Fig. S1). Thus, the oncogene-expressing mammary epithelium in these germline transgenic models exhibits a partial defect in its ability to mount a DDR.

Somatic Activation of PyMT Oncogenic Signaling Induces a Robust DDR in Mouse Mammary Glands. Next, we sought to determine whether acute, somatic activation of an oncogene could engender a robust DDR response. First, we used the potent viral oncogene *PyMT* to provide maximal oncogenic stress in somatic cells, because PyMT promotes hyperproliferation, neoplastic growth, and tumor formation at a rapid rate in mammary epithelial cells (23). We chose two different approaches to express *PyMT* in somatic mammary cells. One model is an inducible transgenic mouse line, in which *PyMT* expression is induced by administration of doxycycline to MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* bitransgenic mice (24). Another model is the RCAS-TVA system, in which mammary epithelial cells in MMTV-*tva* transgenic mice are infected via intraductal injection of the avian retrovirus RCAS carrying *PyMT* (22). One advantage of the RCAS-TVA system is that oncogenes can be introduced into relatively few mammary epithelial cells, which are then allowed to evolve into cancer in the context of a normal mammary gland, thus better recapitulating the etiology of human carcinogenesis than transgenic oncogene models (25, 26). Four MMTV-*tva* and three MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice were exposed to PyMT signaling by intraductal delivery of RCAS-*PyMT* or administration of doxycycline in the diet, respectively, for 1 week at 6 weeks of age to allow early hyperplasias to develop. Early lesions arising in RCAS-*PyMT* and MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mammary cells displayed equivalent oncogene expression based on immunostaining for antibodies against PyMT, and similarly increased mitotic index measured by staining for phospho-histone H3 (Fig. S2 *A* and *B*).

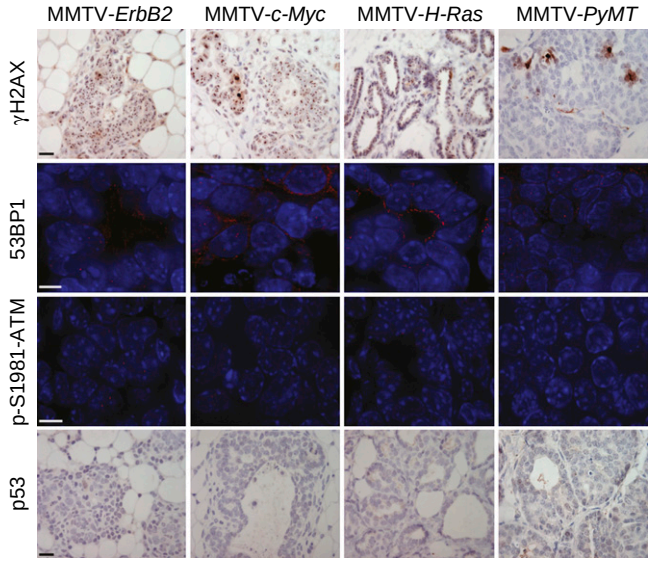

Fig. 1. Poor DDR induction in transgenic mouse models of mammary cancer. Early lesions in mammary glands from 7 to 9-week-old MMTV-*ErbB2* ($n = 4$), MMTV-*c-Myc* ($n = 4$), MMTV-*H-RasG12D* ($n = 3$), and MMTV-*PyMT* ($n = 4$) transgenic mice were stained for the proteins indicated, by immunofluorescence or immunohistochemistry. Brightfield images are 40× with 20 μm scale bar. Deconvolution immunofluorescent images are 100× (53BP1) or 60× (p-S1981-ATM) with 5-μm scale bar.

In these somatic models of oncogenic activation, significant, intense staining of γH2AX foci in the lesions was observed as compared with that in the normal ducts (Fig. S2*A*). There was also marked induction of 53BP1 focal staining in the RCAS-*PyMT* early lesions; however, there was minimal 53BP1 staining in the MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* lesions (Fig. S2*A*). These results indicate that acute induction of PyMT induces a strong DDR in somatic cells, and that this DDR is most readily observed using the RCAS-TVA methodology.

To assess whether activated DDR resulted in stabilization of downstream effector molecules, we examined these early lesions for p53 accumulation using immunohistochemistry. We observed high levels of nuclear p53 staining in the RCAS-*PyMT* early lesions (10.82 ± 1.58%) and only moderate staining in the lesions generated in MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice (2.62 ± 0.21%, $P < 0.05$; Fig. S2 *A* and *C*). To assess the functional activity of the observed p53 stabilization, we examined the level of apoptosis in the early lesions by TUNEL staining. Increased levels of apoptosis were observed in the RCAS-*PyMT* early lesions (3.54 ± 0.48%) with slightly reduced levels in the MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* early lesions (2.17 ± 0.38%, $P < 0.05$; (Fig. S2 *A* and *D*). These data suggest that somatically acquired PyMT signaling causes early mammary lesions with marked induction of a DDR that culminates in the stabilization of the effector protein p53, which presumably suppresses progression to cancer by initiating the apoptotic cascade. Once again, this is most easily observed in the RCAS model of acute oncogene induction.

To confirm that the DDR observed in RCAS-*PyMT* infected glands was not induced by viral infection per se, we injected five MMTV-*tva* mice with RCAS-*GFP* and then harvested the glands 4 days, 1 week, or 2 weeks later for analysis of DDR induction. GFP-positive cells detected at any time point did not exhibit any 53BP1 staining (Fig. S3), indicating that RCAS viral infection itself does not induce a DDR in the target cell, which is consistent with the reported lack of significant production of any viral proteins other than the product from the exogenous gene cloned into this vector (27).

A DDR Persists in RCAS-*PyMT* Induced Tumors. Next, we sought to examine the level of DDR in tumors generated in these two somatic models and, for comparison, in the tumors arising in MMTV-*PyMT* mice. Similar to the early lesions, there was intense focal staining of γH2AX in the RCAS-*PyMT*-induced tumors ($n = 4$), whereas tumors from MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice ($n = 4$) kept on doxycycline had markedly less

(Fig. S4*A*). Similar results were observed with respect to 53BP1 nuclear focal staining (Fig. S4*A*). These results indicate that early DDR sensing remains intact and potent in these two somatic models of tumor induction, especially in the RCAS-TVA model. Tumors arising in MMTV-*PyMT* mice ($n = 3$) did not display dramatic staining of γH2AX or 53BP1 (Fig. S4*A*), consistent with the absence of these DDR markers in the early hyperplastic lesions that develop in this transgenic mouse line.

We then determined whether the DDR signaling cascade in these two somatic models still results in stabilized p53 and apoptosis. We observed abundant nuclear p53 (14.53% ± 3.43%) and TUNEL staining (3.83% ± 0.16%) in the RCAS-*PyMT* tumors (Fig. S4). In contrast, we observed significantly lower levels of p53 (1.19 ± 0.15% and 0.55 ± 0.34%) and TUNEL staining (1.77 ± 0.49% and 1.30 ± 0.21%) in the tumors from MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* mice kept on doxycycline and in the MMTV-*PyMT* tumors ($P < 0.01$ for both, univariate ANOVA; Fig. S4). Therefore, the above data suggest that in the RCAS-*PyMT* model DDR signaling remains fully intact at least in some of the tumor cells.

The observation of high levels of p53 and apoptosis in tumors generated from PyMT-activated somatic mammary cells is contrary to the hypothesis that the DDR must be inactivated for tumors to progress. Because *PyMT* is an extremely potent viral oncogene that can induce tumors with a median latency of approximately 2 weeks in the RCAS-TVA system (22), DDR inactivation may not be required for PyMT-induced tumorigenesis in the mouse mammary epithelium. It is possible that the high rate of cellular proliferation induced by PyMT may overcome the concurrent loss of cells to apoptosis induced by DDR signaling, leading to a rapid net expansion of transformed cells.

A Full DDR Signaling Cascade Is Activated in Response to Aberrant Proliferation Induced by Somatic Activation of ErbB2. ErbB2, which activates many of the same signaling pathways as PyMT, is encoded by a human proto-oncogene that has a well-documented role in breast carcinogenesis (28). Activated ErbB2 in the RCAS-TVA system induces mammary tumors with a median latency of 4 months (22). We therefore asked whether RCAS-*ErbB2* could induce a DDR in hyperplastic lesions, and if so, whether this barrier is overcome in the evolution to cancer.

We injected four 12-week-old MMTV-*tva* mice with RCAS-*ErbB2* (tagged with an HA epitope) and harvested mammary glands 2 weeks later to examine the early precancerous lesions. ErbB2-induced early lesions exhibited focal staining of γH2AX and 53BP1, indicating the presence of a DDR similar to that in RCAS-*PyMT*-induced early lesions (Fig. 2*A*). This was further confirmed by focal staining for p-S1981-ATM (Fig. 2*A*), in a pattern reminiscent of irradiated normal cells. In addition to activated DDR signaling, we also observed ARF induction in ErbB2 early lesions (Fig. 2*A*). Both ATM and ARF regulate oncogene-activated tumor suppressor pathways that converge on p53. p53-positive nuclei were indeed observed in the ErbB2-induced early lesions (6.02% ± 0.92%), and apoptotic cells were readily detected by TUNEL staining (2.24% ± 0.4%), suggesting that p53 is functionally active (Fig. 2). Another endpoint to DDR induction and p53 activation in response to oncogenic stress is cellular senescence (29, 30). To determine whether acute ErbB2-induced DDR activation promotes senescence, we double-stained mammary lesions for senescence-associated β-galactosidase (SA-β-Gal) (31) and for the proliferation marker Ki67. As expected, senescent cells, which stained positively for SA-β-Gal but negatively for Ki67, were readily observed in ≈75% of ErbB2-induced early lesions. This finding was further confirmed by immunostaining for two additional senescence markers, p16 and decoy receptor 2 (DcR2, Fig. 2*A*) (32, 33). These data collectively indicate that acute activation of the *ErbB2* oncogene induces hyperplasias that exhibit DDR signaling and ARF induction, leading to p53 stabilization, apoptosis, and senescence.

Fully Developed Mammary Tumors Induced by Somatic Activation of ErbB2 Retain Upstream DDR Signaling and Senescence but Fail to Up-Regulate p53 or Apoptosis. Mammary tumors that eventually arose in MMTV-*tva* mice infected by RCAS-*ErbB2* also exhibited evidence of DDR signaling, with respect to focal nuclear staining of γH2AX, 53BP1, and p-S1981-ATM, as well as ARF activation ($n = 4$; Fig. 2*A*). However, p53 staining was detected in only very few tumor cells (0.96% ± 0.27%), and this correlated with a very low apoptotic index (0.55 ± 0.25%; Fig. 2). These observations strongly suggest that DDR and ARF functionality at the level of p53 is eventually compromised in ErbB2-induced mammary carcinogenesis, and that these alterations may be required for tumors to arise because of the subsequent loss of apoptosis.

However, to our surprise, positive staining for SA-β-Gal, p16, and DcR2 persisted in many tumor cells (20–40% of cells; Fig. 2*A*). This observation suggests that a p53-independent senescence cascade still remains in some of these tumor cells, although we cannot exclude the possibility that undetectable levels of p53 may still contribute to the senescence observed in these tumor cells.

The down-regulation of p53 was not due to lack of *p53* transcription, as abundant amounts of the *p53* transcript were detected in these tumors ($n = 5$) by qRT-PCR compared with the levels in normal or irradiated spleens (Fig. S5*B*). To test whether this reduced level of p53 resulted from failed stabilization of this very labile gene product, we examined whether p53 could be stabilized by classical DNA damage. We applied γ-radiation (6 Gy) and assayed the tumors ($n = 3$) 16 h later for p53 and its transcriptional targets *Mdm2* and *p21*. p53 was indeed phosphorylated and stabilized upon irradiation (Fig. S5*A*). Both *Mdm2* and *p21* were induced significantly compared with levels in nonirradiated tumors ($n = 5$, $P < 0.05$), and to higher levels than in irradiated spleen controls ($n = 3$; Fig. S5 *C* and *D*). Therefore, in these RCAS-*ErbB2*–induced tumors, the *p53* gene locus appears to be normal, and the product from its transcript can be stabilized and rendered functional after ionizing radiation. Thus, the observed down-regulation of p53 protein levels in RCAS-*ErbB2*-induced tumors likely stems from misregulation at the posttranslational level.

***ATM*-Ablated Mammary Cells Fail to Activate DDR Signaling, Apoptosis, or Senescence in Response to ErbB2-Induced Aberrant Proliferation.** To directly test whether a DDR plays an essential role in activating p53, apoptosis, and senescence under oncogenic stress, we crossed MMTV-*tva* mice into an *ATM*-deficient mouse line (34), and injected 10–12 week-old MMTV-*tva*/*ATM*$^{-/-}$ mice ($n = 3$) with RCAS-*ErbB2* to generate hyperplastic lesions. Heterozygous mice ($n = 3$) and wild-type littermate controls ($n = 3$) were also included. *ErbB2*-expressing early lesions developed in all genotypes (Fig. 3*A*). However, lesions in MMTV-*tva*/*ATM*$^{-/-}$ mice displayed markedly reduced γH2AX and 53BP1 focal staining, indicating diminished DDR signaling in the absence of *ATM* (Fig. 3*A*). As expected, ARF induction as a consequence of ErbB2 oncogenic signaling was not affected by *ATM* status (Fig. 3*A*).

The *ATM*-null lesions exhibited markedly reduced p53 stabilization (2.57% ± 0.92%) and apoptosis (1.4% ± 0.3%) as compared to wild-type controls (p53: 9.79% ± 0.37%; TUNEL: 5.3% ± 0.3%; $P < 0.01$ for both, univariate ANOVA; Fig. 3). Furthermore, *ATM* ablation also resulted in loss of the senescence markers SA-β-Gal and p16 (Fig. 3*A*). These data suggest that ATM, and thus DDR signaling, are required for induction of p53, apoptosis, and senescence following ErbB2 activation, and that ARF induction by itself is not sufficient to stabilize p53 or to induce apoptosis or senescence.

Discussion

DDR signaling is absent at the preneoplastic stage of four different germline transgenic models of breast cancer, and at least in the MMTV-*PyMT* transgenic model, a DDR cannot be robustly activated even by high dose ionizing radiation. In contrast, a potent

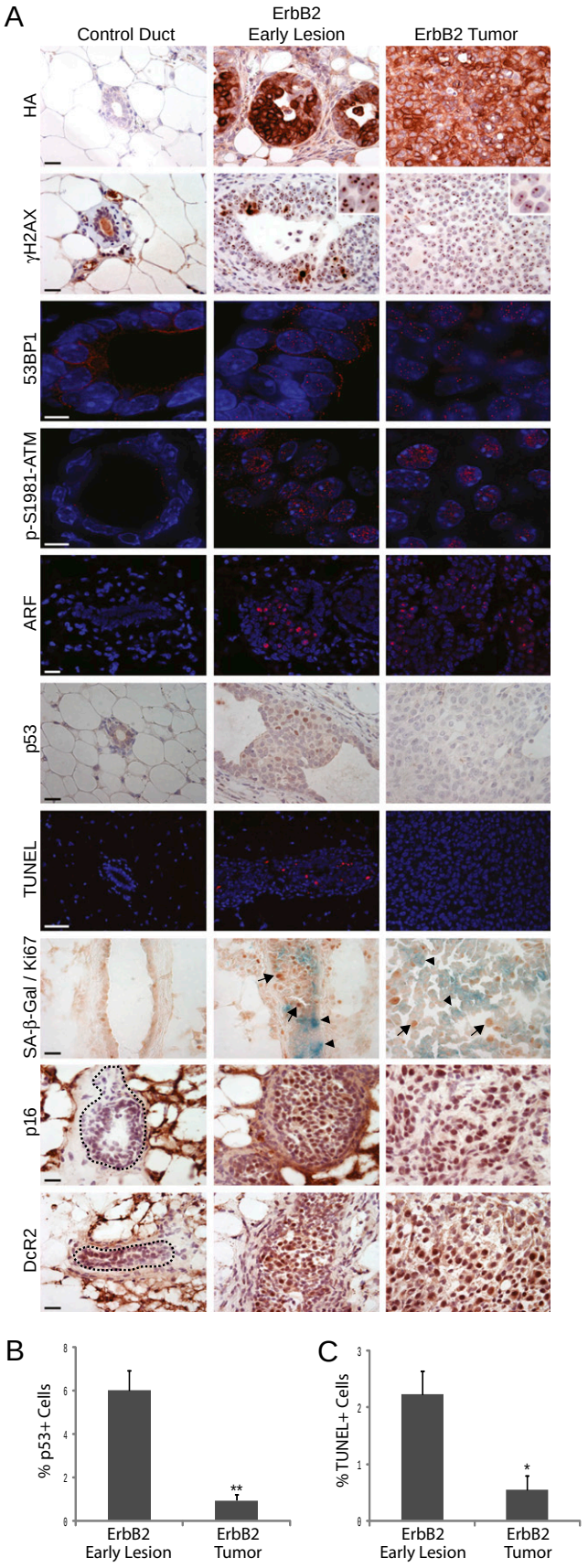

Fig. 2. Somatic activation of ErbB2 induces a DDR that becomes inactivated during tumor progression. (*A*) Normal mammary ducts and hyperplasias in RCAS-*ErbB2*–infected mammary glands from 14-week-old MMTV-*tva* mice ($n = 4$) and mammary tumors from infected mice ($n = 4$) were stained for the proteins indicated and by TUNEL. Arrows indicate Ki67 cells (brown), whereas arrowheads indicate SA-β-Gal-positive cells (blue). Dashed lines, when present, outline normal ducts. Brightfield images are 40× with 20-μm scale bar. 53BP1 and p-S1981-ATM deconvolution immunofluorescent images are 100× with 5-μm scale bar. ARF and TUNEL immunofluorescent images are 40× with 20 μm scale bar. (*B* and *C*) Percentages of p53-positive nuclei (*B*) and TUNEL-positive cells (*C*) in early lesions ($n = 4$) and tumors ($n = 4$) arising in 12-week-old MMTV-*tva* mice injected with RCAS-*ErbB2*. Columns represent mean ± SEM. *$P = 0.01$–0.05; **$P = 0.001$–0.01.

DDR is present in early lesions and persists in tumors in somatic models. These data suggest that the DDR is affected by the developmental stage of the mammary gland that has suffered an oncogenic mutation. One potential explanation for the lack of DDR in these germline models is that oncogene expression during embryonic or prepubertal mammary gland development may have selected for DDR-insensitive cells to populate the mammary fat pad. Alternatively, mammary cells exposed to oncogenic mutations early in development may have adapted to these oncogenes by permanently downregulating the DDR pathway. In support of the latter hypothesis, epithelial cells in embryonic mammary glands have a different transformation response to oncogenic stimuli than cells in more mature mammary glands (35). Nonetheless, models that adopt a germline transgenic approach to oncogene expression appear to be unsuitable for investigating the significance of DDR signaling in human breast cancer initiation, which frequently exhibits DDR signaling. This potential deficiency with germline models may help explain why DDR-mediated anticancer barriers were not detected in some tissues (15).

Somatic introduction of an oncogene, particularly by the RCAS-TVA approach, more closely recapitulates human breast cancer initiation (25, 26). In the inducible PyMT model of breast cancer, a DDR is modestly activated (Fig. S2), whereas RCAS-mediated introduction of PyMT or activated ErbB2 leads to hyperplastic lesions that display components of a potent DDR including γH2AX, 53BP1, and p-S1981-ATM (Fig. 2 and Fig. S2). In these ErbB2-induced early lesions, p53, apoptosis, and senescence are activated (Fig. 2). This is striking, considering that ErbB2 exhibits potent anti-apoptotic functions in numerous studies (36). In the resulting tumors, both p53 and apoptosis are down-regulated, as expected (Fig. 2). However, the *p53* transcript remains induced and its gene product can still be stabilized after γ-irradiation (Fig. S5), presumably by signaling emanating from DSBs, indicating that the diminished p53 in ErbB2-induced tumors is likely caused by the destabilization of this labile protein or posttranslational misregulation. The p53 reduction is very likely responsible for the concurrent disappearance of apoptosis and, thus, for progression to cancer. Inactivated *p53* promotes carcinogenesis in many tissues including the breast (37), and there is a strong correlation between *p53* missense mutations and *ErbB2* alterations in human breast cancers (38). Our data further illustrate the importance of p53 as part of the barrier to tumorigenesis and provide another explanation for frequent p53 alterations in breast cancer patients.

By inducing early lesions in *ATM*-null mice using RCAS-*ErbB2*, we provide compelling evidence that the ATM-mediated DDR signaling pathway is required for p53 stabilization and the erection of both apoptosis and senescence barriers after the activation of ErbB2 signaling. These data are consistent with observations of an important role for DDR in activating p53 and the apoptosis barrier in several other tissues (10, 11), and establish a direct causal relationship between DDR and senescence in vivo. Our observations also reveal a new dimension to ErBB2 signaling, as an overwhelming number of ErbB2 studies in cultured cells failed to detect senescence or apoptosis in ErbB2-activated cells. Because ErbB2 and its signaling pathways

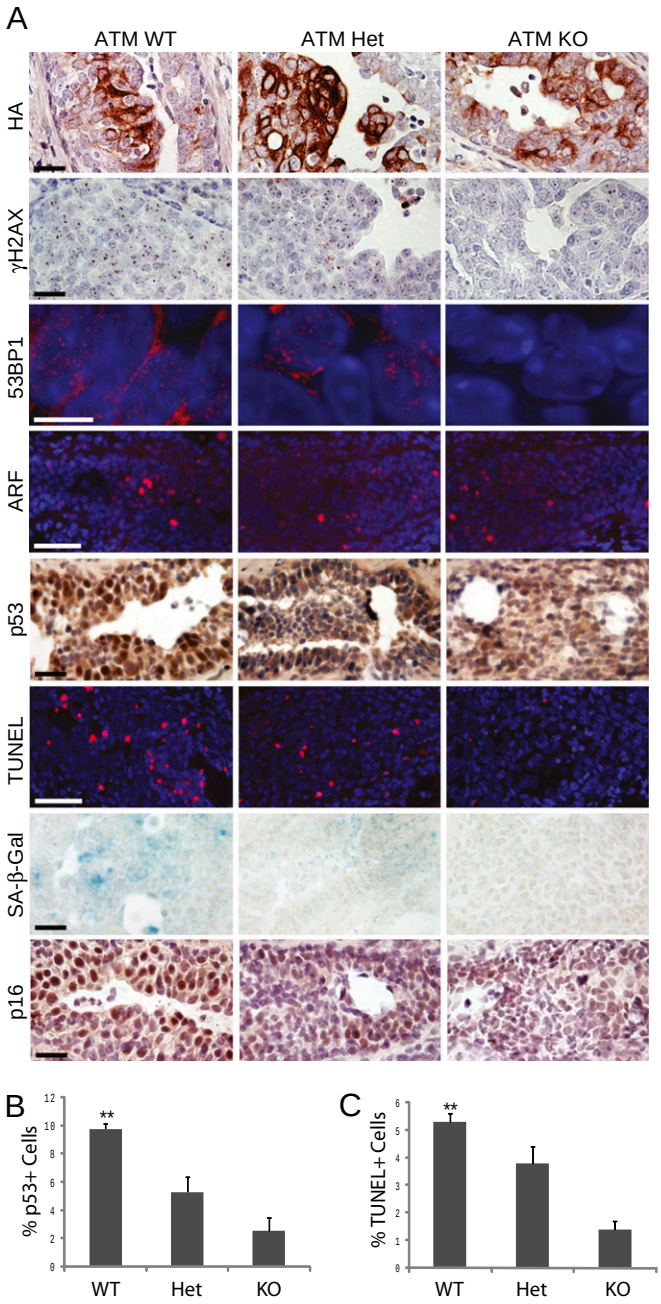

Fig. 3. ATM ablation results in reduced DDR signaling, apoptosis, and senescence in response to ErbB2 activation. (*A*) Hyperplastic mammary glands from RCAS-*ErbB2*–infected 10- to 12-week-old MMTV-*tva* mice with the ATM status indicated at the top were stained for the proteins indicated and by TUNEL. Brightfield images are 40× with 20-μm scale bar. 53BP1 deconvolution immunofluorescent images are 100× with 5 μm scale bar. ARF and TUNEL immunofluorescent images are 40× with 20 μm scale bar. (*B* and *C*) Percentages of p53-positive nuclei (*B*) and TUNEL-positive cells (*C*) in early lesions arising in 10–12 week-old MMTV-*tva*/*ATM*$^{+/+}$ (n = 3), MMTV-*tva*/*ATM*$^{+/-}$ (n = 3), or MMTV-*tva*/*ATM*$^{-/-}$ mice (n = 3) injected with RCAS-*ErbB2*. Columns represent mean ± SEM. Statistical analysis was performed using univariate ANOVA. *P = 0.01–0.05; **P = 0.001–0.01.

are very frequently altered in breast cancers, DDR signaling likely plays a critical role in blocking progression to tumors in at least a substantial proportion of these cases. Therefore, our data potentially provide an explanation for frequent mutations of components of DDR signaling including BRCA1 in breast cancers (39). Furthermore, as ATM plays a critical role in stabilizing p53 in these preneoplastic lesions, genetic or epigenetic alterations that counteract ATM-mediated stabilization of p53 are likely key factors in driving progression of these lesions to malignancy. Unfortunately, *ATM*-null mice succumb to T cell lymphomas and other diseases rapidly, especially after they are crossed to the MMTV-*tva* genetic background (FVB/N), precluding our ability to ascertain that *ATM* loss accelerates ErbB2-driven sporadic mammary tumorigenesis.

ARF is induced in both early lesions and tumors in MMTV-*tva* mice infected by RCAS-*ErbB2* (Figs. 2 and 3). ErbB2 may have induced ARF through its two downstream components Ras and c-Myc, both of which are classical inducers of *ARF* expression (40, 41). At this time, we do not yet know the relative contribution of ARF to p53 induction, apoptosis, and senescence in comparison with ATM in this RCAS-*ErbB2* model. However, our data suggest that ARF activation alone cannot sustain p53 stabilization, apoptosis, or senescence in the absence of ATM-mediated DDR signaling. Although ARF has been reported to be required for Ras-induced senescence in the mammary gland (18, 42), there is little direct evidence in mammary tumorigenesis to link ARF to apoptosis, which our data suggest serves as a critical barrier that needs to be overcome in the progression to cancer, at least when the initiating oncogene is *ErbB2*. Furthermore, the role of ARF in suppressing mammary tumorigenesis is still controversial (18, 42–44).

Senescence continues to be readily detectable in many of the cells in advanced tumors induced by RCAS-*ErbB2* (Fig. 2), although senescence has been considered an important barrier to carcinogenesis (12, 13, 45–47), and has been suggested to be lost in progression to breast carcinomas and other malignancies (18, 32, 48). Residual expression of senescent markers has also been noted in advanced human colon and urinary carcinomas (12). The continued presence of a large fraction of senescent cells in established tumors such as those induced by RCAS-*ErbB2* suggests that in the evolution of some malignancies, abrogation of the senescence response may not be as critical as inactivation of apoptosis. However, it is more probable that the small subset of the ErbB2-activated premalignant cells that failed to activate both senescence and apoptosis eventually evolved into a cancer, and that some of the tumor cells later become senescent. The presence of some senescent tumor cells may also be consequential to a cellular hierarchy (cancer stem cells, progenitor cells, and differentiated cells) that has been found in many malignancies including breast cancer (49, 50). The nonsenescent cancer stem cells may provide the driving force for the expansion of the tumor mass, whereas the senescent cells may be part of the differentiated tumor cells that have exhausted their proliferative potential. The molecular pathway controlling senescence in some of these RCAS-*ErbB2*–induced tumor cells is yet to be defined, although it is likely ATM-dependent. p53, undetectable in the tumors by immunohistochemical staining (Fig. 2), is probably dispensable for the senescence observed, although the undetectable levels of p53 in these tumor cells may still play a role. p53-independent senescence has been observed in Chk2-activated cancer cell lines including those from breast cancer (51, 52). On the other hand, p16^{INK4a}, which has been reported to arrest the cell cycle and to promote senescence (29, 53–55), is present in RCAS-*ErbB2*–induced mammary tumors and may be important in initiating or maintaining senescence (Fig. 2). In addition, Rb, which is another driver of senescence (3), may be activated in these tumor cells to support senescence.

In conclusion, in this in vivo model that recapitulates the DNA damage signaling cascade following somatic oncogenic activation, we demonstrate that, in the mammary gland, ErbB2 activation causes ATM-dependent apoptosis and senescence that cannot be sustained by the concurrent activation of ARF. p53 is deactivated in progression to cancer with simultaneous sup-

pression of apoptosis, while senescence remains in some of the tumor cells. These data provide further mechanistic insights into the frequent deactivation of DDR signaling and p53 in breast cancer, and suggest that protection of the DDR-p53 signaling pathway is important in breast cancer prevention.

Experimental Procedures

Transgenic Mice and Animal Care. MMTV-*tva* (22), MMTV-*PyMT* (56), MMTV-*ErbB2* (57), MMTV-*c-Myc* (58), MMTV-*H-RasG12D* (59), and MMTV-*rtTA*/tet-O-*PyMT-IRES-Luc* (24) mice have been reported. These mouse strains were maintained in an FVB genetic background. To generate MMTV-*tva*/*ATM*$^{-/-}$ mice, MMTV-*tva* mice were crossed to *ATM*$^{+/-}$ mice generously provided by Chengming Zhu (34). The resulting offspring had a mixed FVB/129Sv/C57B6 genetic background. All mouse lines were housed in pathogen-free housing in accordance with National Institutes of Health guidelines.

Virus Preparation and Mammary Gland Delivery. RCAS-*PyMT*, RCAS-*ErbB2*, and RCAS-*GFP* viruses were prepared and injected as described previously (22).

Tissue Harvest and Analysis. Mammary glands and tumors were removed, fixed, and analyzed as described in *SI Experimental Procedures*. Detailed antibody information can be found in *SI Experimental Procedures*.

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1. Halazonetis TD, Gorgoulis VG, Bartek J (2008) An oncogene-induced DNA damage model for cancer development. *Science* 319:1352–1355.
2. Venkitaraman AR (2005) Medicine: aborting the birth of cancer. *Nature* 434:829–830.
3. Lowe SW, Cepero E, Evan G (2004) Intrinsic tumour suppression. *Nature* 432:307–315.
4. Bartek J, Bartkova J, Lukas J (2007) DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene* 26:7773–7779.
5. Kastan MB (2008) DNA damage responses: Mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture. *Mol Cancer Res* 6:517–524.
6. Harper JW, Elledge SJ (2007) The DNA damage response: Ten years after. *Mol Cell* 28: 739–745.
7. Kastan MB, Bartek J (2004) Cell-cycle checkpoints and cancer. *Nature* 432:316–323.
8. Bartkova J, et al. (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434:864–870.
9. Gorgoulis VG, et al. (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 434:907–913.
10. Reimann M, et al. (2007) The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. *Blood* 110:2996–3004.
11. Pusapati RV, et al. (2006) ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. *Proc Natl Acad Sci USA* 103:1446–1451.
12. Bartkova J, et al. (2006) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444:633–637.
13. Di Micco R, et al. (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444:638–642.
14. Mallette FA, Gaumont-Leclerc MF, Ferbeyre G (2007) The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev* 21:43–48.
15. Liao MJ, Yin C, Barlow C, Wynshaw-Boris A, van Dyke T (1999) Atm is dispensable for p53 apoptosis and tumor suppression triggered by cell cycle dysfunction. *Mol Cell Biol* 19:3095–3102.
16. Efeyan A, et al. (2009) Limited role of murine ATM in oncogene-induced senescence and p53-dependent tumor suppression. *PLoS One*, 4:e5475, 1-11.
17. Woditschka S, Haag JD, Sullivan R, Gould MN (2009) A short-term rat mammary carcinogenesis model for the prevention of hormonally responsive and nonresponsive in situ carcinomas. *Cancer Prev Res* 2:153–160.
18. Sarkisian CJ, et al. (2007) Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nat Cell Biol* 9:493–505.
19. Ayyanan A, et al. (2006) Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc Natl Acad Sci USA* 103:3799–3804.
20. Umesako S, et al. (2005) Atm heterozygous deficiency enhances development of mammary carcinomas in p53 heterozygous knockout mice. *Breast Cancer Res* 7: R164–R170.
21. Lu S, et al. (2006) Atm-haploinsufficiency enhances susceptibility to carcinogen-induced mammary tumors. *Carcinogenesis* 27:848–855.
22. Du Z, et al. (2006) Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models. *Proc Natl Acad Sci USA* 103:17396–17401.
23. Marcotte R, Muller WJ (2008) Signal transduction in transgenic mouse models of human breast cancer—implications for human breast cancer. *J Mammary Gland Biol Neoplasia* 13:323–335.
24. Podsypanina K, et al. (2008) Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* 321:1841–1844.
25. Du Z, Li Y (2007) RCAS-TVA in the mammary gland: An in vivo oncogene screen and a high fidelity model for breast transformation? *Cell Cycle* 6:823–826.
26. Reddy JP, Li Y (2009) The RCAS-TVA system for introduction of oncogenes into selected somatic mammary epithelial cells in vivo. *J Mammary Gland Biol Neoplasia* 14:405–409.
27. Hughes SH (2004) The RCAS vector system. *Folia Biol (Praha)* 50:107–119.
28. Ursini-Siegel J, Schade B, Cardiff RD, Muller WJ (2007) Insights from transgenic mouse models of ERBB2-induced breast cancer. *Nat Rev Cancer* 7:389–397.
29. Campisi J, d'Adda di Fagagna F (2007) Cellular senescence: When bad things happen to good cells. *Nat Rev Mol Cell Biol* 8:729–740.
30. Di Micco R, Fumagalli M, d'Adda di Fagagna F (2007) Breaking news: High-speed race ends in arrest—how oncogenes induce senescence. *Trends Cell Biol* 17:529–536.
31. Dimri GP, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA* 92:9363–9367.
32. Collado M, et al. (2005) Tumour biology: Senescence in premalignant tumours. *Nature* 436:642.
33. Collado M, Serrano M (2006) The power and the promise of oncogene-induced senescence markers. *Nat Rev Cancer* 6:472–476.
34. Borghesani PR, et al. (2000) Abnormal development of Purkinje cells and lymphocytes in Atm mutant mice. *Proc Natl Acad Sci USA* 97:3336–3341.
35. Andrechek ER, Hardy WR, Laing MA, Muller WJ (2004) Germ-line expression of an oncogenic erbB2 allele confers resistance to erbB2-induced mammary tumorigenesis. *Proc Natl Acad Sci USA* 101:4984–4989.
36. Hynes NE, Lane HA (2005) ERBB receptors and cancer: The complexity of targeted inhibitors. *Nat Rev Cancer* 5:341–354.
37. Donehower LA (1996) The p53-deficient mouse: A model for basic and applied cancer studies. *Semin Cancer Biol* 7:269–278.
38. Langerød A, et al. (2007) TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. *Breast Cancer Res*, 9:R30, 1-16.
39. Deng CX, Wang RH (2003) Roles of BRCA1 in DNA damage repair: A link between development and cancer. *Hum Mol Genet* 12(Spec No 1, Spec No 1)R113–R123.
40. Palmero I, Pantoja C, Serrano M (1998) p19ARF links the tumour suppressor p53 to Ras. *Nature* 395:125–126.
41. Zindy F, et al. (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 12:2424–2433.
42. Swarbrick A, Roy E, Allen T, Bishop JM (2008) Id1 cooperates with oncogenic Ras to induce metastatic mammary carcinoma by subversion of the cellular senescence response. *Proc Natl Acad Sci USA* 105:5402–5407.
43. D'Amico M, et al. (2003) The role of Ink4a/Arf in ErbB2 mammary gland tumorigenesis. *Cancer Res* 63:3395–3402.
44. Debies MT, et al. (2008) Tumor escape in a Wnt1-dependent mouse breast cancer model is enabled by p19Arf/p53 pathway lesions but not p16 Ink4a loss. *J Clin Invest* 118:51–63.
45. Michaloglou C, et al. (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 436:720–724.
46. Chen Z, et al. (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436:725–730.
47. Braig M, et al. (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 436:660–665.
48. Trost TM, et al. (2005) Premature senescence is a primary fail-safe mechanism of ERBB2-driven tumorigenesis in breast carcinoma cells. *Cancer Res* 65:840–849.
49. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. *Nat Rev Cancer* 8:755–768.
50. Rosen JM, Jordan CT (2009) The increasing complexity of the cancer stem cell paradigm. *Science* 324:1670–1673.
51. Aliouat-Denis CM, et al. (2005) p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res* 3:627–634.
52. Chen CR, et al. (2005) Dual induction of apoptosis and senescence in cancer cells by Chk2 activation: Checkpoint activation as a strategy against cancer. *Cancer Res* 65: 6017–6021.
53. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88:593–602.
54. Beauséjour CM, et al. (2003) Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO J* 22:4212–4222.
55. Narita M, et al. (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113:703–716.
56. Guy CT, Cardiff RD, Muller WJ (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* 12:954–961.
57. Guy CT, et al. (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* 89: 10578–10582.
58. Leder A, Pattengale PK, Kuo A, Stewart TA, Leder P (1986) Consequences of widespread deregulation of the c-myc gene in transgenic mice: Multiple neoplasms and normal development. *Cell* 45:485–495.
59. Sinn E, et al. (1987) Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: Synergistic action of oncogenes in vivo. *Cell* 49:465–475.

Appendix B

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The RCAS-TVA System for Introduction of Oncogenes into Selected Somatic Mammary Epithelial Cells in Vivo

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Abstract We have reported the use of the RCAS-TVA system to model sporadic tumorigenesis upon oncogenic activation in somatic mammary epithelial cells in the mouse. Here we review the advantages of this approach as compared to conventional mouse models with transgenic oncogene expression. We also in detail describe the RCAS-TVA method for introducing genes into somatic mammary epithelial cells engineered to express the avian receptor *tva*. This method may be particularly useful in modeling oncogenic activation and subsequent tumorigenesis in distinct breast epithelial cell sub-populations, including progenitor cells.

Keywords Mammary gland · Breast cancer · Somatic cell · tva · RCAS

Abbreviations

ALV	avian leukosis virus
MMTV	mouse mammary tumor virus
RCAS	replication competent ALV-LTR, splice receptor
TVA	tumor virus A

Introduction

In recent years, many mammary gland biologists have sought ways to better model sporadic mammary tumorigenesis, which comprises the majority of breast cancers in humans. Transgenic expression of oncogenes or germline ablation of tumor suppressor genes remain oft-used, powerful tools to study tumor initiation and progression. However, there are important limitations to these approaches: It is difficult to target a small number of cells within a given tissue (as opposed to all cells in the tissue, as in transgenic or knockout models) to model sporadic carcinogenesis, and the development of genetically-engineered mouse models requires significant investments of time and money.

To circumvent these issues, viral vectors have been used to introduce specific genetic lesions (alone and in combination) into a small number of cells in vivo [1, 2]. This approach has the advantage of allowing the determination of the impact of activating an oncogene (or Cre-mediated deletion of a loxP-flanked tumor suppressor) in a given cell. One such system, the RCAS-TVA method, is particularly useful in modeling cancer in a variety of tissues. It was first reported by Federspiel and colleagues [3] that murine myoblasts engineered to express a transgene encoding the avian viral receptor TVA could selectively be infected by viral vectors of the avian leukosis virus subgroup A (ALV-A). A few years later, this method was adapted for oncogene introduction to a number of murine tissues, first into the brain [4, 5], and then into the ovary [6], pancreas [7–9], liver [10], vascular endothelium [11–13], and the mammary gland [14, 15]. This approach relies on the coupling of transgenic expression of *tva* to a specific tissue or cell type of interest (via the use of appropriate transgenic promoters) with the introduction of ALV-A vectors encoding a gene of interest.

TVA is a glycoprotein conserved in avian species that enables the cellular entry of an ALV-A retrovirus. *tva* is not expressed in mammalian cells, but ectopic expression of *tva*, especially in a specific tissue courtesy of tissue-specific promoters, enables efficient in vivo infection by ALV-A-based viruses (Fig. 1). As mentioned above, this basic premise has been adapted to allow the successful infection by ALV-based viruses of several murine tissues with transgenic *tva* expression. Thus far, several transgenic mouse lines expressing *tva* in the mammary gland have been generated. Use of the mouse mammary tumor virus (MMTV) promoter allows *tva* expression in approximately 50% of the mammary epithelium and can be used to introduce candidate genes into a heterogeneous population

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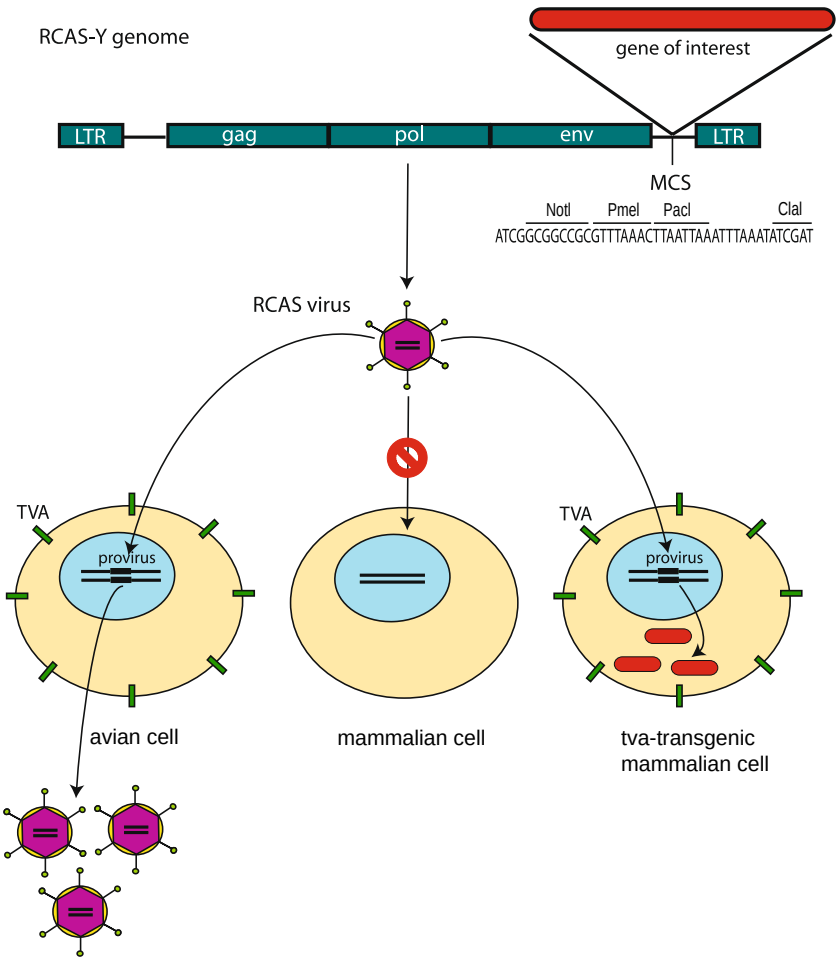

Figure 1 The RCAS-TVA system. A gene of interest can be inserted into the RCAS viral vector at the splice acceptor site behind the endogenous viral genes. Mammalian cells cannot be infected unless *tva* is expressed. Transgenic expression of *tva* with selected promoters in mice can restrict viral infection to specific cell or tissue types. Upon infection, only the exogenous gene is transcribed—gag, pol, and env are not expressed for unknown reasons. The map is not drawn to scale.

of mammary epithelial cells [14]. Additional lines that allow for *tva* expression in distinct mammary epithelial cell populations have also been developed and are described as follows: *keratin 19-tva* drives *tva* expression in luminal epithelial cells [15, 16], *keratin 5-tva* restricts *tva* expression to basal cells and potentially stem cells as well [6], *whey acidic protein (WAP)-tva* is expressed in differentiated mammary cells (M.J.T. and Y.L. unpublished), and *keratin 6-tva* allows for infection of keratin 6-positive cells which may represent a mammary progenitor cell population (W.B. and Y.L unpublished). Each line has the advantage of targeting distinct mammary cell populations with a defined genetic lesion and modeling subsequent mammary cancer initiation and progression (Fig. 2). Infection of tva-expressing cells requires ALV-A-based retroviral vectors termed RCAS (replication competent ALV-LTR splice acceptor). As implied by their name, these are derivatives of ALV viruses that contain the requisite genes to achieve competency of produc-

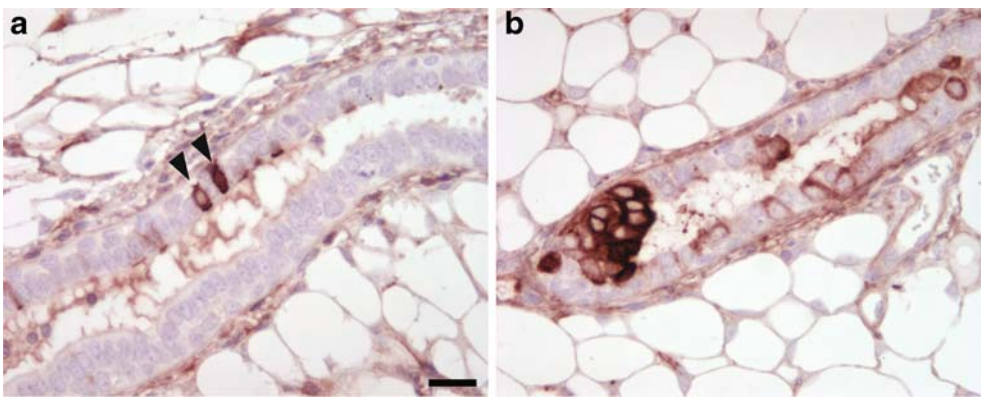

Figure 2 Mammary epithelial cells infected with RCAS-*β-actin* (A) or RCAS-*ErbB2* (B). Pubertal MMTV-*tva* mice were injected with RCAS-*β-actin-HA* or RCAS-*ErbB2*-HA and then examined 1 week later. Arrowheads indicate infected cells expressing ectopic β-actin. RCAS-*ErbB2*-infected cells had expanded and invaded the ductal lumen. Scale bar is 20 μm.

tive infection and have been engineered to contain splice acceptor sites to accommodate exogenous genes, the expression of which is then driven by the viral LTR [17].

Efficient in vivo infection of specific mammary epithelial cell populations can be achieved by introducing an RCAS viral preparation containing an exogenous gene of interest into the mammary gland of a mouse engineered to express *tva*. This unique approach represents a powerful method to model sporadic breast cancer initiation and progression. Specific experimental guidelines and techniques are described below.

Methods

Construction of Viral Vector

Many variants of RCAS viral vectors have been developed with varying advantages and disadvantages (for a comprehensive list of RCAS variants, please visit http://home.ncifcrf.gov/hivdrp/RCAS/). For in vivo infection of murine somatic mammary cells in vivo, the RCASBPA (Replication Competent ASLV-LTR Splice acceptor, Bryan Polymerase, sub-group A) vector is preferred as this particular viral construct can achieve the requisite high-titer production of mature virions for intraductal injection into the murine mammary gland [18]. Furthermore, RCASBPA possesses a Rous sarcoma virus LTR that can achieve improved exogenous gene expression in mammary epithelial cells (MECs) in vivo. A single restriction enzyme site (ClaI) with RCASBP can be used for insertion of a cDNA of interest [19]. (An additional ClaI site is present but is subject to *dam* methylation and, thus, can be ignored since *dam*+ bacteria are routinely used in propagation of RCASBP viral cDNAs.) Needless to say, the presence of only one site for subcloning is inconvenient, so RCASBPA variants have been constructed. For example, RCASBPA has been modified to contain a NotI, PmeI, SwaI, PacI, as well as the original ClaI site in the multiple cloning site (MCS) region — this variant is hereafter termed RCAS-Y, or simply RCAS (Fig. 1).

Careful consideration should be taken when constructing an RCAS vector with an exogenous gene in the splice acceptor site. A practical size limitation of 3-kb exists, as the insertion of exogenous cDNAs larger than this leads to poor titer yields when collecting viral particles [17]. Large RCAS viral genomes do not package properly into mature virions and as a result lead to inefficient viral replication as compared to empty RCAS vectors which replicate with high efficiency. Inserts less than 2.5-kb can be reliably cloned into RCAS with the eventual creation of a high titer viral yield. Inserts between 2.5- and 3-kb may or may not be successful depending on the insert. We have found that inserts that fall into this range may lead to an unstable viral genome leading to truncation of the cDNA insert to enable efficient viral replication (J.P.R. and Y.L, unpublished observations). Within the size limitations, it is possible to clone into RCAS two separate, relatively short cDNAs that are linked by an IRES element to generate expression of both target genes in infected cells. However, this approach often results in reduced expression of both genes, the products of which become more difficult to detect using in situ assays such as immunohistochemistry.

Once constructed, care should be taken when preparing large amounts of RCAS cDNA for transfection. The presence of the appropriate cDNA insert and absence of contaminating RCAS vectors should be confirmed by digestions with several diagnostic restriction enzymes. Contamination by additional, smaller viral vectors that more efficiently replicate in avian cells inevitably leads to the harvest of an undesired viral preparation or low titer of the desired viral preparation.

Viral Production

To achieve high titer viral production, the immortalized cell line DF-1 is commonly used [20–22]. This line is derived from chicken fibroblasts and has endogenous *tva* expression rendering it susceptible to productive infection by RCAS virions. The DF-1 cell line is available from the ATCC (CRL-12203). Specific procedures for viral production are detailed below.

1. Maintain uninfected DF-1 cells in standard cultured conditions (DMEM with high glucose, 10% fetal bovine serum, 2 mM L-glutamine, 10 units/ml penicillin, and 10 µg/ml streptomycin; 37°C)
2. Passage them to several 60 mm dishes with 20–50% confluency.
3. Transfect cells with 5 µg of RCAS vector of interest using standard lipofection-based approaches, e.g. Superfect (Qiagen), Fugene 6 (Roche). It is generally recommended to include a non-transfected control plate as well as a positive control plate transfected with an RCAS vector carrying a fluorescent reporter, e.g. green fluorescent protein (GFP).
4. At 24–48 h post-transfection, assess transfection efficiency in the positive control dish using an inverted fluorescent microscope. Transfection efficiency should be above 50%.
5. With several passages, DF-1 cells should achieve 100% infection. Initially transfected cells will generate mature RCAS virions that will infect neighboring cells. Tip: if the transfected positive control cells fail to achieve 100% expression of GFP, this indicates that a contaminating RCAS virus is present, interfering with

subsequent infection with the virus of interest. To avoid cross-contamination, it is generally recommended that different viruses be handled in the tissue culture hood sequentially, allowing at least 5-minute hood time between viruses.

6. Once DF-1 cells have achieved 100% infection (as assessed by monitoring of the positive control), passage them to eighteen 15-cm dishes for the eventual harvesting of viral medium.
7. Once these DF-1 cells have become confluent, replace the medium with the minimal amount of fresh growth medium (~12 ml) to cover the cell monolayer.
8. 24 hrs later, collect the viral medium from each of the plates into several tubes and centrifuge at 3500 rpm for 5 min at 4°C to remove cell debris.
9. Carefully remove the supernatant from each tube, place it 30-ml ultracentrifuge tubes, and spin at 125000xg for 90 min at 4°C. A small pellet with viscous consistency should be observed. Resuspend the pellet in the growth medium at 1/100th of the original volume, combine the concentrated viruses into a single tube, aliquot, and store at −80°C.
10. Collect another batch 24 h later — this process may be repeated for up to a week.
11. The infected DF1 cells may be frozen in 10% DMSO and 20% FBS in liquid nitrogen for future viral production. It is recommended that these producer cells be frozen down within a week of transfection to avoid potential contamination.

Additional Notes: The viral titer may have variability between daily preparations. If consistent viral titers are necessary for mouse infection over a period of time, it is important to thaw one aliquot from each day of viral collection, combine them, and use this mixed content for each time, and for viral titering as well (see below). In this manner, the overall titer of the viral batch can remain consistent throughout one's experiments. It is also important to note that repeated freeze-thaw cycles of concentrated virus is not recommended as each cycle may reduce the effective viral titer by as much as 10-fold.

Viral Titer Determination

1. Passage DF-1 cells to several 60 mm dishes. Culture them to achieve 30–50% confluence. Replace the growth medium with 2 ml fresh growth medium.
2. Take one frozen aliquot from each day of viral collection, combine the contents, make serial dilutions (10^{o} to 10^{11}), and add 1 ml from each dilution point into a dish. Return the dishes to the incubator.
3. After they reach confluency, passage the cells to a new set of 60 mm dishes. Discard excess cells. This passaging is important to maintain the cells in logarithmic growth, so that the virus can rapidly spread.
4. After cells have reached confluency from the second passage, lyse cells using standard protocols (e.g., by scraping with 0.5 ml RIPA buffer supplemented with protease inhibitors). Perform Western blotting on cell lysates for the protein of interest. The titer in infectious units per ml is the highest dilution of the concentrated virus that leads to a detectable specific band. Of note, other assays, such as immunofluorescent staining or PCR-based methods, may also be used to detect the virus-encoded protein product or the provirus in these infected cells. Typical titers in the concentrated stock range from 10^{9} to 10^{11}. Viral preparations with lower titers result in too few detectable infected cells in vivo, and should be discarded.

In Vivo Infection of Murine Mammary Epithelial Cells with RCAS Virus

Efficient in vivo infection of mammary epithelial cells requires flooding the mammary ductal tree with a high titer RCAS viral preparation by performing intraductal injection. This method has been described in detail previously but will be briefly reviewed here in the context of RCAS viral delivery [23].

The technique of intraductal technique requires precise movements, thus, the mouse must be completely immobi-

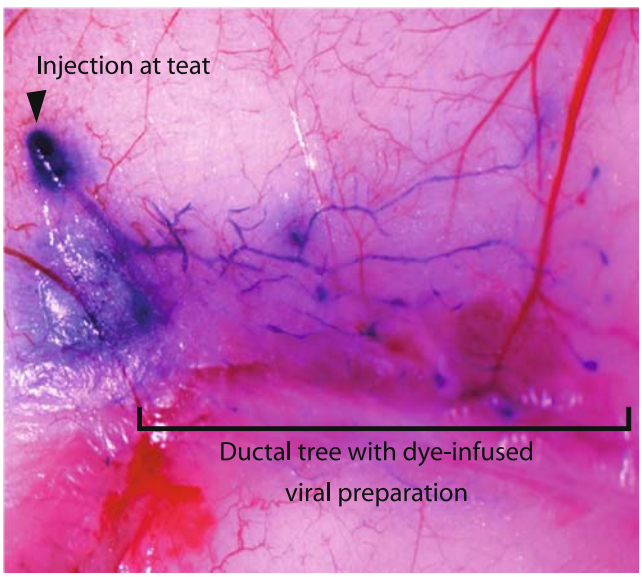

Figure 3 Intraductal injection of a mouse mammary gland with a dye-infused RCAS viral preparation. A Hamilton syringe containing RCAS viral preparation with bromophenol blue was injected into the murine lactiferous duct via the teat. Injection of virus can be visualized by observing the blue dye permeating the mammary ductal tree. The virus is capable of reaching and infecting cells in TEBs [1].

lized by anesthetization. Once anesthetized, spread out the mouse fore- and hind-limbs on a flat surface to increase the surface area of the abdomen. The teats should be easily observed in this posture, but to ease visualization ethanol may be used to move adjacent hair and expose the teat. Remove the tip of the teat using fine micro-dissecting scissors (Roboz) to expose an opening that can be used to introduce the viral preparation. It is recommended that #2, 3, and 4 mammary glands be used for injection as these are typically easier to inject. The #1 and 5 mammary glands can be injected but are less ideal due to smaller nipple size and less epithelial content.

Add a sesame seed-sized amount of bromophenol blue powder to the freshly thawed concentrated virus to make a dark blue solution. Use a 50-μl syringe with a 33 gauge metal hub needle (Hamilton) to carefully inject the virus into the open nipple duct. The dye enables visualization of a successfully performed injection, as one can observe the dye permeating the mammary ductal tree (Fig. 3). Volumes from 10 μl to 30 μl can be injected without rupturing the ductal tree. Larger volumes may be used but may cause ductal damage due to increased pressure. Injections should be performed slowly to minimize damage caused by rapidly moving fluid within ductal lumens.

In our laboratory, injection of 10 μl of high titer virus (a total of 10^8 IUs) into 8–12 week-old MMTV-*tva* mice results in approximate 0.3% of infected cells when assayed 4 days post infection [14]. Similar rates of infection can be achieved in *keratin 19-tva* or WAP-*tva* mice, but much fewer cells are infected in *keratin6-tva* due to the much lower number of susceptible cells in this line.

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References

1. Du Z, Li Y. RCAS-TVA in the mammary gland: an in vivo oncogene screen and a high fidelity model for breast transformation? Cell Cycle. 2007;6(7):823–6.
2. Gould MN. The introduction of activated oncogenes to mammary cells in vivo using retroviral vectors: a new model for the chemoprevention of premalignant and malignant lesions of the breast. J Cell Biochem Suppl. 1993;17G(66–72):66–72.
3. Federspiel MJ, Bates P, Young JA, Varmus HE, Hughes SH. A system for tissue-specific gene targeting: transgenic mice susceptible to subgroup A avian leukosis virus-based retroviral vectors. Proc Natl Acad Sci U S A. 1994;91(23):11241–5.
4. Holland EC, Varmus HE. Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice. Proc Natl Acad Sci U S A. 1998;95(3):1218–23.
5. Holland EC, Li Y, Celestino J, Dai C, Schaefer L, Sawaya RA, et al. Astrocytes give rise to oligodendrogliomas and astrocytomas after gene transfer of polyoma virus middle T antigen in vivo. Am J Pathol. 2000;157(3):1031–7.
6. Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE. Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. Cancer Cell. 2002;1(1):53–62.
7. Lewis BC, Klimstra DS, Varmus HE. The c-myc and PyMT oncogenes induce different tumor types in a somatic mouse model for pancreatic cancer. Genes Dev. 2003;17(24):3127–38.
8. Du YC, Lewis BC, Hanahan D, Varmus H. Assessing tumor progression factors by somatic gene transfer into a mouse model: Bcl-xL promotes islet tumor cell invasion. PLoS Biol. 2007;5(10):2255–69.
9. Seidler B, Schmidt A, Mayr U, Nakhai H, Schmid RM, Schneider G, et al. A Cre-loxP-based mouse model for conditional somatic gene expression and knockdown in vivo by using avian retroviral vectors. Proc Natl Acad Sci U S A. 2008;105(29):10137–42.
10. Lewis BC, Klimstra DS, Socci ND, Xu S, Koutcher JA, Varmus HE. The absence of p53 promotes metastasis in a novel somatic mouse model for hepatocellular carcinoma. Mol Cell Biol. 2005;25(4):1228–37.
11. Montaner S, Sodhi A, Molinolo A, Bugge TH, Sawai ET, He Y, et al. Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. Cancer Cell. 2003;3(1):23–36.
12. Sausville J, Molinolo AA, Cheng X, Frampton J, Takebe N, Gutkind JS, et al. RCAS/SCL-TVA animal model allows targeted delivery of polyoma middle T oncogene to vascular endothelial progenitors in vivo and results in hemangioma development. Clin Cancer Res. 2008;14(12):3948–55.
13. Vervoort VS, Lu M, Valencia F, Lesperance J, Breier G, Oshima R, et al. A novel Flk1-TVA transgenic mouse model for gene delivery to angiogenic vasculature. Transgenic Res. 2008;17(3):403–15.
14. Du Z, Podsypanina K, Huang H, McGrath A, Toneff MJ, Bogoslovskaia E, et al. Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models. Proc Natl Acad Sci U S A. 2006;103(46):17396–401.
15. Siwko S, Bu W, Gutierrez C, Lewis BC, Jechlinger M, Schaffhausen B, et al. Lentivirus-mediated oncogene introduction into mammary cells in vivo induces tumors. neoplasia. 2008;11(7):653–62.
16. Morton JP, Mongeau ME, Klimstra DS, Morris JP, Lee YC, Kawaguchi Y, et al. Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. Proc Natl Acad Sci U S A. 2007;104(12):5103–8.
17. Hughes SH. The RCAS vector system. Folia Biol (Praha). 2004;50(3–4):107–19.
18. Petropoulos CJ, Hughes SH. Replication-competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. J Virol. 1991;65(7):3728–37.
19. Hughes SH, Greenhouse JJ, Petropoulos CJ, Sutrave P. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J Virol. 1987;61(10):3004–12.
20. Kim H, You S, Kim IJ, Farris J, Foster LK, Foster DN. Increased mitochondrial-encoded gene transcription in immortal DF-1 cells. Exp Cell Res. 2001;265(2):339–47.
21. Himly M, Foster DN, Bottoli I, Iacovoni JS, Vogt PK. The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. Virology. 1998;248(2):295–304.
22. Schaefer-Klein J, Givol I, Barsov EV, Whitcomb JM, VanBrocklin M, Foster DN, et al. The EV-O-derived cell line DF-1 supports the efficient replication of avian leukosis-sarcoma viruses and vectors. Virology. 1998;248(2):305–11.
23. Nguyen D-A, Beeman N, Lewis M, Schaack J, Neville MC. Intraductal injection into the mouse mammary gland. In: Ip MM, Asch BB, editors. Methods in Mammary Gland Biology and Breast Cancer Research. New York: Kluwer Academic; 2000. p. 259–70.